Molecular genetic characterisation of the Asc locus of tomato conferring resistance to the fungal pathogen Alternaria alternata f. sp. lycopersici

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Molecular genetic characterisation of the *Asc* locus of tomato conferring resistance to the fungal pathogen *Alternaria alternata* f. sp. *lycopersici*

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Key words: AAL-toxins, *Alternaria alternata*, *Asc* locus, *Lycopersicon esculentum*, positional cloning, transposon tagging

Abstract

The Alternaria stem canker disease of tomato is caused by the fungal pathogen *Alternaria alternata* f. sp. *lycopersici* and its host-selective AAL-toxins. Resistance to the pathogen and insensitivity to the toxins are conferred by the *Asc* locus on chromosome 3L. Sensitivity to AAL-toxins is a relative character; the toxins inhibit development of all tested tomato tissues but susceptible cultivars are much more sensitive than resistant cultivars. In addition to tomato, some other plant and animal species are sensitive to the toxins as well. The likely mode of action of AAL-toxins is interference with sphingolipid biosynthesis by specific inhibition of ceramide synthase activity. To molecularly isolate *Asc*, transposon tagging and positional cloning strategies are applied. As a first step, transposon insertions and restriction fragment length polymorphism (RFLP) markers are identified in proximity of the *Asc* locus. Subsequently, the transposons are used to inactivate *Asc* by insertion mutagenesis, and the RFLP markers are used to identify yeast artificial chromosomes (YACs) with tomato DNA inserts. Once an *Asc*-insertion mutant and/or a YAC encompassing *Asc* has been obtained, physical isolation and characterisation of *Asc* will be conceivable. Elucidation of the molecular role of *Asc* will illuminate the specificity of host recognition by *Alternaria alternata* f. sp. *lycopersici*.

Abbreviations: AAL-toxin – *Alternaria alternata lycopersici*-toxin; *A. a. lycopersici* – *Alternaria alternata* f. sp. *lycopersici*; Asc – Alternaria stem canker; HST – host-selective toxin

Introduction

Understanding the molecular specificity of host recognition by plant pathogens offers an attractive challenge for phytopathologists. Although plants are continuously exposed to potential parasites, only a few infect their hosts. The molecular mechanisms underlying the specificity of these successful host-pathogen interactions remain to be solved. However, the basis of host recognition is generally unpredictable because the pathogens have multiple effects on the host cells, and several host strategies are employed to prevent pathogen infection. Additionally, the dominant or recessive nature of inheritance of a resistance locus has little bearing on the function of the gene products involved. Therefore, the physical isolation and characterisation of resistance and susceptibility genes will greatly contribute to an understanding of the specificity of host recognition.

Present host plants and fungal pathogens are supposedly the result of co-evolution: through parasitic adaptation, necrotrophic fungi developed to plant pathogens. The lowly adapted facultative saprophytes kill host cells by toxins and obtain the nutrients from dead cells, while the highly adapted obligate biotrophic parasites achieve nutrients from living host cells (Heath, 1987). It is anticipated that for these different types of pathogens, plants developed different defence strategies (De Wit, 1992; Walton & Panaccione, 1993). The biotrophic pathogens illustrate gene-for-gene rela-

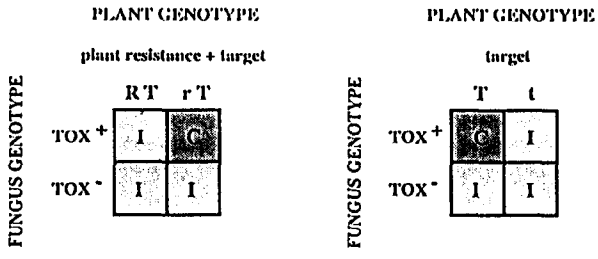


Fig. 1. Plant-fungal HST interaction models. In both cases the target for the HST is encoded by the T locus. Plants with an active defence mechanism (left) have a resistance locus R that acts upon the HST rendering it nonactive and, consequently, have an incompatible interaction (I) with the fungus. Absence of the resistance locus (r) results in the inability to induce a resistance response and, hence, in a compatible interaction (C). Plants that do not have a defence mechanism at their disposal (right) can resist fungal infection (I) if they also lack the target locus (t). Plants that do contain the T locus are susceptible to fungal infection (C) (modified from Heath, 1993). *Abbreviations*: T – target present, t – target absent, R – resistance present, r – resistance absent, I – incompatible interaction, C – compatible interaction.

tionships by releasing elicitors which are specifically recognised by the hosts that subsequently activate resistance responses. Host susceptibility in these cases results from the inability to respond with a resistance reaction and is inherited as a recessive trait (e.g. tomato-*Cladosporium fulvum*, Van den Ackerveken et al., 1992).

Different plant defence strategies operate against saprophytic pathogens. Some strains of the saprophytic fungi *Cochliobolus* (*Helminthosporium*) and *Alternaria alternata* secrete metabolic compounds that are selectively toxic to their corresponding hosts (monocots and dicots, respectively). These host-selective toxins (HSTs) display the same host specificity as the pathogen itself and are the primary pathogenicity factors (Otani & Kohmoto, 1992; Walton & Panaccione, 1993). Research on the effects of the HSTs from these pathogens resulted in accumulating support that susceptibility can, apart from the inability to induce a resistance response, also be a consequence of the lack of a resistance mechanism (Fig. 1, right). This situation is illustrated by two host-*Cochliobolus* interactions; 1) *C. heterostrophus* secretes T-toxin causing southern corn leaf blight in cms-T lines of maize (*Zea mays*). Susceptibility is maternally inherited because the biochemical target for T-toxin, the URF13 protein, is mitochondrially encoded. Some maize lines do not have a resistance mechanism but simply lack the gene coding for URF13, and therefore, are resistant to fungal infection (Levings III, 1991; Levings III & Siedow, 1992). 2) *C. victoriae* produces victorin (HV-toxin) that causes victoria blight in oats (*Avena sativa*). The product of the *Vb* gene is assumed to be involved in formation of the target of victorin. Lines containing *Vb* are susceptible to pathogen infection, and therefore susceptibility is inherited as a dominant trait (Wolpert & Macko, 1991; Akimitsu et al., 1993). Host susceptibility, in these cases, is a genotype specific property and relies on the presence of susceptibility genes (*urf13* or *Vb*) which encode targets for the HSTs (T-toxin or victorin). The resistant hosts lack the targets or have targets with low affinity to HSTs and thus, active resistance mechanisms are absent.

Conversely, hosts that do harbour functional resistance mechanisms respond with active strategies, e.g. HST detoxification (Fig. 1, left). This possibility is exemplified by *C. carbonum* that produces HC-toxin and causes leaf spot in susceptible maize lines. The dominantly inherited HM1 gene encodes an enzyme that chemically modifies HC-toxin. The target for HC-toxin is not known yet (Meeley et al., 1992; Johal & Briggs, 1992).

The fungal pathogen *Alternaria alternata* f. sp. *lycopersici* secretes host-selective AAL-toxins that are involved in the molecular recognition of susceptible hosts. Only tomato (*Lycopersicon esculentum*) genotypes harbouring the *Alternaria stem canker* (*Asc*) locus are insensitive to the toxins and, hence, resistant to fungal infection. This paper deals with the progress in understanding the molecular specificity of host recognition by the pathogen that is determined by the two alleles of the *Asc* locus.

Alternaria stem canker disease of tomato

The Asc locus

The Alternaria stem canker disease of tomato and the causal fungus, *Alternaria alternata* f. sp. *lycopersici*, were first reported in 1975 in California, USA (Grogan et al., 1975). The typical symptoms are slowly enlarging dark-brown-to-black cankers on the stems near the soil line or aboveground. Topmost leaflets develop necrotic areas between the veins or, in later stages, become completely necrotic (Fig. 2). Associated with the disease are the AAL-toxins, produced by the fungus, which can be isolated from diseased plants and elicit identical symptoms to those that develop on nat-

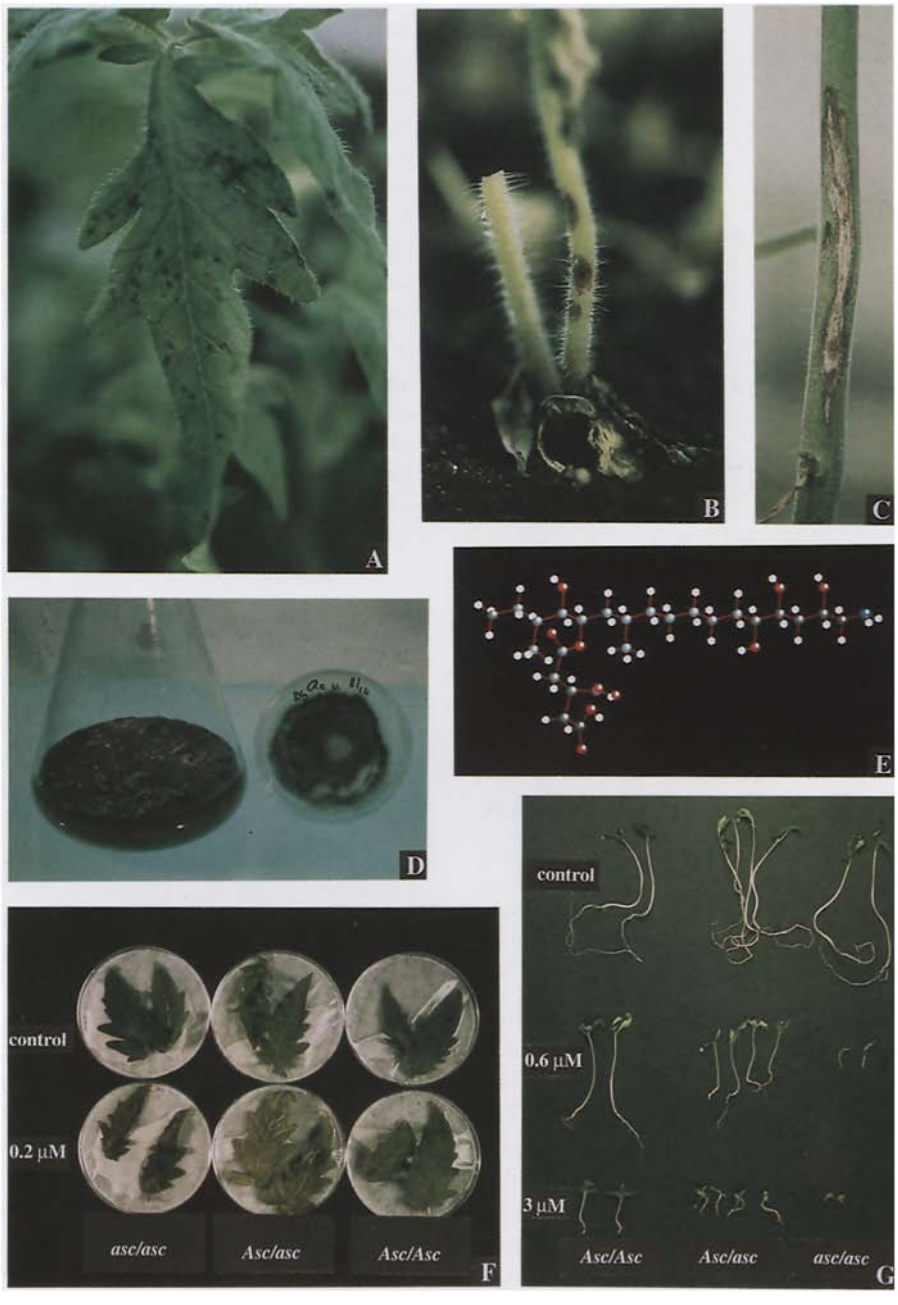


Fig. 2. Interaction between tomato *Asc* genotypes and *Alternaria alternata* f. sp. *lycopersici* and its host-selective AAL-toxins. (A) Necrotic lesions on a leaflet from a susceptible cultivar (*asc/asc*) infected with fungal spores; (B, C) Alternaria stem cankers of infected susceptible plants (*asc/asc*) at the seedling and the mature stage, respectively; (D) Fungal cultures growing on liquid (left) and solid (right) media; (E) Computer prediction of the three-dimensional structure of AAL-toxins (white = hydrogen, grey = carbon, red = oxygen, blue = nitrogen); (F) Necrosis on leaflets of the three *Asc* genotypes in bioassays using 0.2 μM AAL-toxins (see text for description); (G) Inhibited development of seedlings of the three *Asc* genotypes germinated in the presence of 0.6 μM and 3 μM AAL-toxins; controls contain water only.

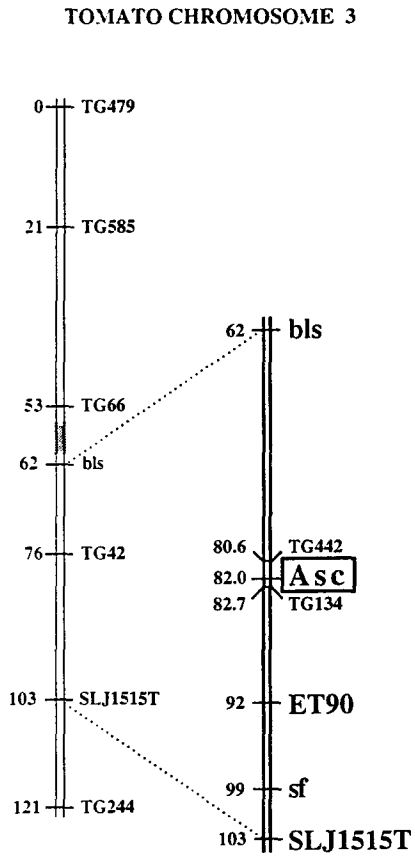


Fig. 3. Genetic map of the long arm of chromosome 3 containing the *Asc* locus, some RFLP markers and the morphological markers *bls* (baby lea syndrome) and *sf* (solanifolia). Distances are expressed in cM, the grey rectangle denotes the centromere. SLJ1515T and ET90 are T-DNA insertions carrying an *Ac* and a *Ds* element, respectively.

urally infected plants (Gilchrist & Grogan, 1976; Siler & Gilchrist, 1983). In 1977 the disease was detected in Japan in susceptible tomatoes and shown to be caused by the same fungal species (Kohmoto et al., 1982). The pathogen was also identified in other parts of the USA (Abbas & Vesonder, 1993; Abbas et al., 1993b), and in Southern Europe (Smith et al., 1988). It is not known whether the occurrence in the different geographical areas resulted from independent development of new pathotypes or introduction of the pathotype into another region.

Following the identification of susceptible and resistant tomato lines, the segregation of resistance was investigated. One single locus with two alleles segregated in a Mendelian fashion and was designated *Asc* for *A*lternaria *s*tem *c*anker (Gilchrist & Grogan, 1976). Heterozygotes (*Asc/asc*) show intermediate phenotypes in AAL-toxin sensitivity assays, but can fully resist fungal infection. Therefore, the *Asc* locus is dominant for resistance to infection by the pathogen but semi-dominant for insensitivity to AAL-toxins. Near-isogenic *Asc* lines (NILs) were made by continuous selfing (F_9) of heterozygotes and have proven to be very useful in characterising the sensitivity to AAL-toxins (Clouse & Gilchrist, 1987). Genetic mapping with morphological markers revealed *Asc* to be flanked by *solanifolia* (*sf*) and *baby lea syndrome* (*bls*) on the long arm of chromosome 3 (Witsenboer et al., 1989; Van der Biezen et al., 1994b). Because *Asc* has not been introgressed from wild relatives and there are virtually no DNA polymorphisms among tomato cultivars, the resistant wild tomato *L. pennellii* was crossed with a susceptible line. The interspecific progenies segregated for resistance and permitted restriction fragment length polymorphism (RFLP) analysis. Chromosome 3 specific RFLP markers (Tanksley et al., 1992) placed *Asc* on the middle of chromosome 3L (Fig. 3) (Van der Biezen et al., 1994a).

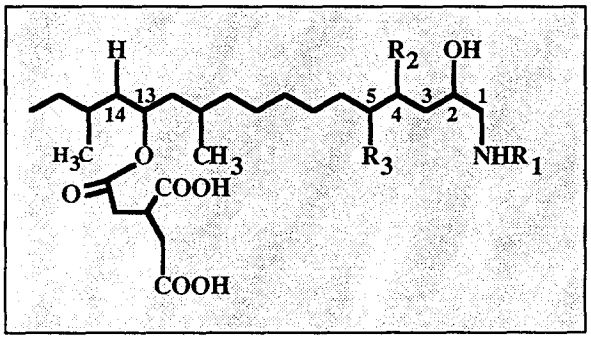
Alternaria and its AAL-toxins

Alternaria alternata (Fries) Keissler (*A. tenuis* Nees) is a widespread saprophytic filamentous fungus that is commonly found in the soil and on agricultural products after harvest (Tiejin & Ceponis, 1982). The species is composed of many strains (*formae speciales*) that are classified according to morphological similarities at the asexual stage (Simmons, 1967). Seven strains are known that, in addition to a saprophytic existence, also infect and colonise specific plant species and, therefore, are referred to as pathotypes of *A. alternata* (Otani & Kohmoto, 1992). These facultative saprophytes are distinguished by their host range and produce different HSTs which, in most cases, consist of multiple closely related molecules (Table 1). Frequently, *A. alternata* isolates spontaneously lose their ability to produce toxins. The toxin-less mutants simultaneously fail to infect their host plants and become indistinguishable from strains that are exclusively saprophytic. Two tomato diseases are known that are caused by *A. alternata*: 1) blackmold, a ripe tomato fruit rot caused by a saprophytic form (Grogan et al., 1975; Pearson & Hall, 1975), and 2) Alternaria stem canker, caused by *A. alternata* f. sp. *lycopersici* that affects tomato stems, leaves and green fruits (Grogan et al., 1975).

A. alternata f. sp. *lycopersici* has a limited host range. Following inoculation of 265 tomato cultivars

Table 1. The *Alternaria alternata* pathotypes with their host-selective toxins and the disease of the susceptible hosts.

Pathotype	Host-selective toxins	Disease
Apple	AM-toxin I, II, III	Alternaria blotch
Japanese pear	AK-toxin I, II	Alternaria black spot
Rough Lemon	ACRL-toxin I	Alternaria brown spot
Strawberry	AF-toxin I, II, III	Alternaria black spot
Tangerine	ACT-toxin A, B	Alternaria brown spot
Tobacco	AT-toxin	Alternaria brown spot
Tomato	AAL-toxin T_A, T_B, T_C, T_D, T_E	Alternaria stem canker



TOXIN	R_1	R_2	R_3
T_A	H	OH	OH
T_B	H	OH	H
T_C	H	H	H
T_D	C (=0) CH_3	OH	H
T_E	C (=0) CH_3	H	H

Fig. 4. Chemical structure of the AAL-toxins. The 17-carbon backbone of all AAL-toxins is the 1-amino-dimethyl-heptadecapentol (upper panel). The AAL-toxin analogues T_A, T_B, T_C, T_D, and T_E differ with respect to the attached molecules at C-1 (R_1), C-4 (R_2) and C-5 (R_3) (lower panel). Only the structures of the AAL-toxin isomers-1 are shown. Isomers-2 have opposite groups at the C-13 and C-14 positions, i.e. hydrogen at C-13 and propane-tricarboxylic acid at C-14.

with a spore suspension, 25% were susceptible to pathogen infection (Grogan et al., 1975). Infection assays of divergent families showed all tested plant species to be resistant to the fungus and insensitive to the AAL-toxins (Gilchrist & Grogan, 1976). Therefore, tomato was initially thought to be the only species susceptible to *A. a. lycopersici* infection. However, examination of additional species showed that *Lycopersicon cheesmanii*, a wild tomato relative from the Galápagos Islands, was susceptible to fungal infection (Van der Biezen et al., 1994a). The AAL-toxins possess typical host-selective characteristics: in all cases tested a positive correlation was found between susceptibility to fungal infection and sensitivity to AAL-toxins.

The chemical structure of AAL-toxins was elucidated by mass spectroscopy and nuclear magnetic resonance (NMR) spectrometry. AAL-toxins are 1-amino-dimethyl-heptadecapentols esterified to propane-tricarboxylic acid with an average molecular weight of 521 Da (Fig. 2). The toxins consist of at least five related molecules: T_A, T_B, T_C, T_D, and T_E, and each toxin itself consists of two isomers (Fig. 4) (Bottini & Gilchrist, 1981; Bottini et al., 1981; Clouse et al., 1985; Caldas et al., 1994). The T_A and T_B analogues have similar specific activity which is 30–400 times higher than that of the other forms (Caldas et al., 1994). Therefore, only T_A and T_B toxins have been used in experiments described here and are referred to as AAL-toxins. Concentrations of AAL-toxins are determined by a quantitative chemical assay using high performance liquid chromatography (HPLC) (Siler & Gilchrist, 1982). Determination of the three-dimensional structure and synthetic production of AAL-toxins are in progress (Oikawa et al., 1994). The fungus maintains its capacity to produce toxins when cultured on various artificial media. Different amounts of toxins are produced, however, among different culture substrates and different fungal isolates

Table 2. Physiological effects of AAL-toxins on tomato *Asc* genotypes.

Tissues/cells/organelles	[AAL](μM)	Physiological effect	*Asc/Asc*	*Asc/asc*	*asc/asc*	reference[2]
Calli	0.1–1	browning, growth inhibition	+[1]	nd	++	(4)(8)(9)
Cell suspensions	3	growth inhibition, reduced viability	+	nd	++	(2)(4)(8)
Fruits	0.02	necrotic lesions	-	nd	+	(3)
Leaf discs	0.02/1	shoot induction inhibition/necrotic lesions	+	nd	++	(8)(9)
Leaf endoplasmatic reticula	20	swollen, vesiculated	-	nd	+	(6)
Leaf mitochondria	20	swollen, leached matrix, reduction of cristae	-	nd	+	(6)
Leaf protoplasts	10	reduced viability	+	++	+++	(5)(10)
Leaves	1	necrotic lesions	+	++	+++	(5)(7)(10)
Pollen	60	germination and tube growth inhibition	+	nd	++	(1)
Roots	0.2	growth inhibition	+	++	+++	(7)(8)(9)

[1] - = no effect; +, ++ and +++ = relative differences of effect; nd = not determined

[2] (1) Bino *et al.*, 1988; (2) Fuson & Pratt, 1988; (3) Gilchrist *et al.*, 1992; (4) Kodama *et al.*, 1991; (5) Moussatos *et al.*, 1993b; (6) Park *et al.*, 1981; (7) Van der Biezen *et al.*, 1994a; (8) Witsenboer *et al.*, 1988; (9) Witsenboer *et al.*, 1989; (10) Witsenboer *et al.*, 1992

(Siler & Gilchrist, 1983; Gilchrist et al., 1992; Abbas & Vesonder, 1993; Shepard et al., 1993).

Sensitivity to AAL-toxins

The AAL-toxins have been invaluable for the characterisation of the tomato-*A. a. lycopersici* interaction. The responses to AAL-toxins were studied of various tissues from resistant (*Asc/Asc*), susceptible (*asc/asc*) and heterozygous (*Asc/asc*) genotypes (Fig. 2). The physiological effects of the toxins include 1) development of necrotic lesions on leaves and fruits; 2) inhibition of *in vitro* development of calli, pollen, roots and shoots; and 3) reduced viability of protoplasts and suspension cells (Table 2). It has been demonstrated that AAL-toxins inhibit plant cell development at various levels of differentiation, that sensitivity to AAL-toxins is present at vegetative and generative tissues, and that *Asc* is expressed at the level of an individual cell. The concentrations of AAL-toxins necessary to cause symptom development vary for the different tissues, indicating differences in effectiveness of the toxins. All tissues showed similar genotype-specific differential responses with the resistant tissues being less sensitive than the susceptible tissues. For tissues from plants that are heterozygous for *Asc*, intermediate responses to AAL-toxins were demonstrated. The relative insensitivity of these heterozygotes (*Asc/asc*) can be overcome by application of a high concentration of AAL-toxins. Accordingly, the toxin concentration employed in the bioassays determines the sensitivity responses to AAL-toxins and, hence, the mode of inheritance of insensitivity. Insensitivity to AAL-toxins inherits as a (semi)dominant trait when low toxin concentrations are used, when high concentrations are applied insensitivity inherits in a recessive fashion.

For sake of sensitivity and simplicity, leaflet bioassays are most often applied for the determination of sensitivity to AAL-toxins (Fig. 2). After incubation of detached leaflets on filter papers saturated with a dilution of AAL-toxins in sealed petridishes, the level of necrosis is assessed (Clouse & Gilchrist, 1987). Because susceptibility to fungal infection has until now always been correlated with sensitivity to AAL-toxins, leaflet bioassays are also performed for plant genotyping. The percentage of necrosis is directly proportional to the concentration of AAL-toxins, and the duration of exposure to AAL-toxins. Therefore, the leaves are exposed to specific concentrations (usually 0.2 μM) and during defined periods (usually 72 h). In addition, the leaves need to be exposed to light for a minimum of 12 h to allow necrosis to develop (Witsenboer et al., 1992; Moussatos et al., 1993b). Finally, aging plays a role, indicating a developmental regulation of *Asc* sensitivity. The youngest leaflets on a leaf are about 3 times more sensitive then the older following leaflets (Moussatos et al., 1993b).

In the physiological complexity of AAL-toxin-treated tomato tissues, it is difficult to distinguish causes from effects. Moreover, the conclusions are limited of experiments that did not include time-response and AAL-toxin dosage-response courses. The physiological studies have shown the consequences of the AAL-toxins rather than their mode of action. To deter-

mine the cause of toxicity to sensitive tissue, and to explain the mechanism of insensitivity to the toxins and resistance to the fungus, the biochemical target of the AAL-toxins needs to be revealed.

Targets of AAL-toxins

Before necrotic symptoms can be detected on detached leaflets of a susceptible line, AAL-toxins at low concentration cause a specific decline in the sucrose uptake capacity, and an accumulation of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), followed by an increase in the production of ethylene (Moussatos et al., 1993a, 1994). Using exogenous supplied ACC and inhibitors of endogenous ACC, a correlation between ethylene production and AAL-toxin-induced necrosis was demonstrated. In addition, it was observed that the pyrimidine precursor dihydro-orotic acid (DHO) reduced the AAL-toxin-induced necrosis on tomato leaflets, possibly by suppression of ACC synthesis. From these results it was hypothesised that the action of *Asc* is linked to sucrose transport, ethylene biosynthesis, pyrimidine metabolism and cell death (Moussatos et al., 1994). The suggestion that AAL-toxins disrupt pyrimidine metabolism by inhibiting the activity of aspartate carbamoyltransferase (ACTase) (Gilchrist, 1983) could not be confirmed experimentally (Fuson & Pratt, 1988; Kodama et al., 1991; Abbas et al., 1992). In addition, it was demonstrated that *Asc* does not encode ACTase (Overduin et al., 1993).

By analysing the contents of 18 amino acids and 5 related compounds in leaves from infected plants and in AAL-toxin-treated leaves, a specific accumulation of ethanolamine (EA) and phosphoethanolamine (PEA) was observed (Kawaguchi et al., 1991). Since EA and PEA are the primary and secondary intermediate metabolites of the phospholipid biosynthesis pathway in higher plants, the possible interference of AAL-toxins with this pathway was further investigated. Following application of ^{14}C-EA precursor to susceptible AAL-toxin-treated leaf discs, strong inhibition of the incorporation of EA into phosphatidylethanolamine (PtdEA) was observed (Orolaza et al., 1992). Therefore, enzymes of the phospholipid pathway were suggested as potential biochemical targets for AAL-toxins.

The involvement of AAL-toxins in sphingolipid biosynthesis was found following experiments with fumonisins (reviewed in Abbas et al., 1993a; Norred, 1993; Riley et al., 1993; Merrill et al., 1993). Fumonisins are a group of toxins that share structural homology with AAL-toxins and were first identified in cultures of the fungus *Fusarium moniliforme* (Sheldon) and, later, also in *A. a. lycopersici* cultures (Bezuidenhout et al., 1988; Gelderblom et al., 1988; Chen et al., 1992). *F. moniliforme* and, hence, the fumonisins are a common contaminant of maize grain (*Zea mays*) throughout the world. Fumonisins are not acutely toxic to maize itself, but are carcinogenic in laboratory rats and cause identical symptoms to domestic animals as various diseases attributed to *F. moniliforme*-contaminated feed. Associated with human consumption of infected grains is a higher incidence of esophageal cancer. Results of physiological and biochemical experiments indicate that AAL-toxins and fumonisins have a similar mode of action. Both toxins have the same spectrum of phytotoxicity: they elicit similar genotype-specific symptoms in tomato (Gilchrist et al., 1992; Mirocha et al., 1992) and have identical effects on jimsonweed (*Datura stramonium*), black nightshade (*Solanum nigrum*) (Abbas et al., 1992, 1993b), and duckweed (*Lemna pausicostata* and *L. minor*) (Vesonder et al., 1992a; 1992b; Tanaka et al., 1993). In addition, fumonisins and AAL-toxins showed to be toxic to rat liver and dog kidney cells (Shier et al., 1991; Mirocha et al., 1992; Vesonder et al., 1993).

The suggested mechanism of action of AAL-toxins and fumonisins was derived from the observation that these compounds bear structural similarity to sphinganine, a primary constituent of sphingolipids (Fig. 5). It was observed in rat hepatocytes that the toxins inhibit the incorporation of ^{14}C-serine into sphingosine and that the level of ^{14}C-sphinganine increased. Subsequently, it was shown that the fumonisins and the AAL-toxins disrupt *de novo* sphingolipid biosynthesis by inhibiting ceramide synthase (sphinganine *N*-acyltransferase) (Wang et al., 1991; Merrill et al., 1993). From their structural resemblance to sphinganine it was suggested that fumonisins and AAL-toxins are recognised by ceramide synthase as substrate analogues. As a result of the inhibition of this enzyme, the ceramide biosynthesis decreases, leading to the accumulation of sphinganine and a depletion of complex sphingolipids in several mammals, higher plants and yeast (*Pichia ciferri*) (Kaneshiro et al., 1992, 1993; Merrill et al., 1993; Riley et al., 1993). Sphingolipids have numerous functions in controlling cell behaviour, including the regulation of cell receptors and cell growth, differentiation, cell-cell communications, stabilisation of membranes, sorting of lipids and proteins, and interactions with cytoskeletal elements (Hannun & Bell, 1989; Shier, 1992). Evidently, AAL-

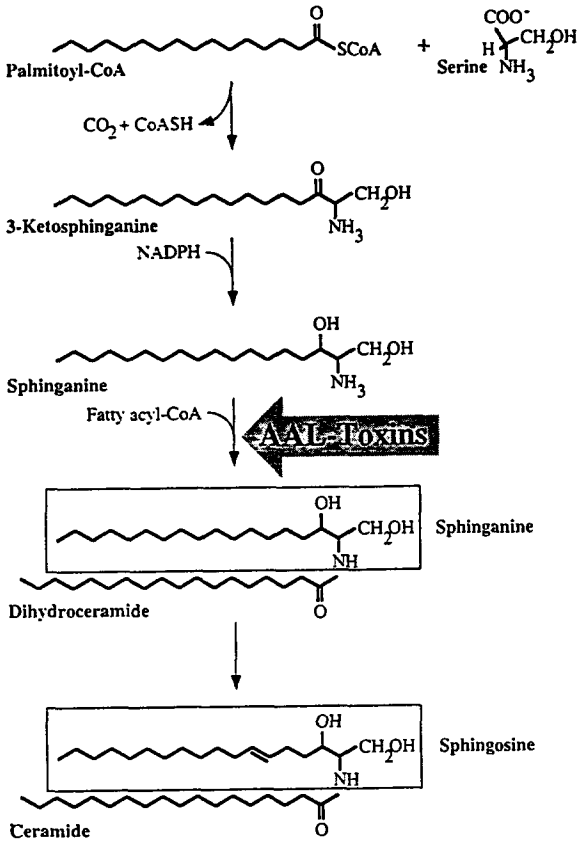


Fig. 5. Sphingolipid biosynthesis pathway and site of action of AAL-toxins and fumonisins (after Merrill et al., 1993). AAL-toxins inhibit ceramide synthase (sphinganine *N*-acyltransferase), leading to accumulation of sphinganine and reduced synthesis of complex sphingolipids.

toxins would have a wide spectrum of effects at a wide range of tissues which is validated by the observed physiological responses.

The genotype-specific differential sensitivity of resistant and susceptible tomato lines that is regulated by the different alleles of the *Asc* locus remains unclear. It can be postulated that host resistance or susceptibility is due to chemical modification of the AAL-toxins. In explaining resistance, the dominant *Asc* allele might be involved in the detoxification of AAL-toxins. Following modification, the toxins are not recognised by ceramide synthase as substrate analogues and, hence, have lost their toxicity. Conversely, to explain susceptibility, the recessive *asc* allele might code for ceramide synthase that upon inactivation by the AAL-toxins leads to cell death. Another option is that the AAL-toxins themselves are harmless but are modified to toxic metabolites by an *asc*-encoded enzyme. In the latter two cases, resistance would be based on the inability to recognise or convert AAL-toxins. Alternatively, both the *Asc* alleles could encode ceramide synthase with differential affinity to AAL-toxins; the dominant allele (conferring resistance) having less affinity to AAL-toxins than the recessive allele (conferring susceptibility). This suggestion also explains the relative action of *Asc* (Van der Biezen et al., 1993). These hypotheses may be tested by incubation of ^{14}C-AAL-toxins with extracts from resistant and susceptible tomato leaves and subsequent determination of the structural consequences (Meeley et al., 1992; Alberts et al., 1993). As an alternative to these biochemical approaches, the function of *Asc* could also be clarified by following a molecular genetic approach.

Molecular genetic characterisation of the *Asc* locus

Transposon tagging of the Asc locus

For the isolation of genes that are only known by their phenotype several procedures are applicable, including transposon tagging and positional cloning. Both strategies have successfully been used in tomato for the isolation of genes involved in resistance to pathogens (Martin et al., 1993; Jones et al., 1994). For effective transposon mutagenesis several criteria have to be met (Van der Biezen et al., 1994c). First, inactivation of the target gene should result in a recognisable phenotype. With respect to the *Asc* locus, however, the outcome of insertional mutagenesis cannot be predicted. Considering the hypotheses mentioned in the last paragraph explaining the mode of resistance and susceptibility, either one or both *Asc* alleles could be active. Inactivation of the *Asc* or *asc* allele would consequently lead to loss of resistance or loss of susceptibility, respectively. Therefore, two independent transposon tagging experiments were conducted, each aiming at the inactivation of a different *Asc* allele. The second parameter in transposon tagging involves the observed small genetic distance of transposition. The efficiency of transposon mutagenesis can therefore be increased about 100-fold by starting the experiment with a transposon close to the gene-of-interest. In collaboration with several laboratories, numerous transposon-containing T-DNA insertions were mapped on the tomato genome. The two T-DNAs that mapped closest to *Asc* were chosen for transposon tagging of this locus: SLJ1515T

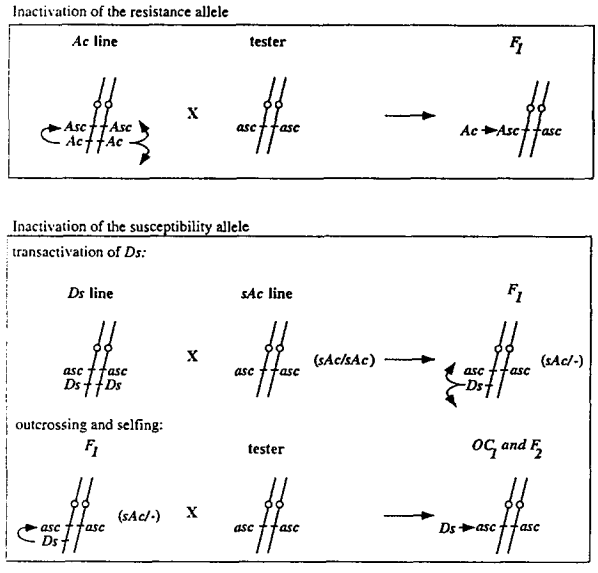


Fig. 6. Transposon tagging directed to different alleles of the *Asc* locus. *Upper panel*: Inactivation of resistance. Following crossing of the resistant (*Asc/Asc*) *Ac* line to the susceptible tester (*asc/asc*), heterozygous (*Asc/asc*) progenies are tested for lost resistance (i.e. susceptibility) as a result of insertional inactivation of the dominant *Asc* allele. *Lower panel:* Inactivation of susceptibility. First, the closely linked *Ds* transposon is transactivated by the stabilised *Ac* element (*sAc*). Subsequently, the F_1 is selfed and outcrossed. The OC_1 and F_2 progenies are screened for lost susceptibility (i.e. resistance) as a result of inactivation of the recessive *asc* allele.

(Thomas et al., 1994) and ET90 (K. Theres, personal communication; Knapp et al., 1994) (Fig. 3).

SLJ1515T contains an autonomous *Ac* element and was located 21 cM distal of *Asc*. Because this *Ac* element had been introduced in an *A. a. lycopersici* resistant line (*Asc/Asc*), it was used for inactivation of the dominant *Asc* allele (Overduin et al., in preparation) (Fig. 6). The *Ac* line was crossed to a susceptible cultivar (*asc/asc*) and heterozygous progenies (*Asc/asc*) were screened for loss of resistance as a result of transposon insertion into the dominant *Asc* allele. Out of approximately 20,000 progenies, one plant was identified that was susceptible to *A. a. lycopersici* infection and contained the original T-DNA and several transposed *Ac* elements. However, association of a specific *Ac* insertion with susceptibility to fungal infection could not yet be demonstrated. Resolving the origin of the susceptibility of this plant is hampered by the fact that its progeny segregates for resistance. This remarkable mutant is currently being further characterised.

To test the hypothesis that the recessive *asc* allele is required for susceptibility to *A. a. lycopersici* infection, a random chemical mutagenesis was conducted (Van der Biezen et al., in preparation). Seeds of a susceptible line (*asc/asc*) were treated with ethyl methanesulfonate (EMS), and after selfing, the M_2 progenies were screened for insensitivity to AAL-toxins. In addition to numerous plants with mutant morphological phenotypes, plants showing insensitivity to AAL-toxins were recovered with high frequency. No differences in the necrotic response to AAL-toxins were observed in leaves from these mutagenised and regular resistant lines (*Asc/Asc* and *Asc/asc*). The mutants were resistant to fungal infection following inoculation with a spore suspension. Allelic tests and genetic mapping of 9 independently derived resistant mutants demonstrated that the recessive *asc* allele was mutated to dominant alleles. Therefore, it is concluded that the recessive *asc* allele confers susceptibility to *A. a. lycopersici*. This EMS mutagenesis approach to obtain resistance to *A. a. lycopersici* resembles the somaclonal variation-based techniques employed for inducing resistance. Resistance against various pathogens in diverse crops was obtained by tissue culture and *in vitro* selection techniques, e.g. resistance against *Fusarium oxysporum* f. sp. *lycopersici* in tomato (Shahin and Spivey, 1986; reviewed in Van den Bulk, 1991). With respect to other *A. alternata* pathotypes, resistance and insensitivity to AK- and AM-toxins have been induced by γ-ray irradiation of Japanese pear and apple (Tabira et al., 1993), and to AT-toxins by *in vitro* selection of tobacco protoplasts (Thanutong et al., 1983).

The results of the EMS mutagenesis of *asc* indicate that resistant plants can potentially also be obtained by transposon mutagenesis. However, the nature of an EMS-induced mutation differs from a transposon-induced mutation. A basepair alteration (EMS) in the *asc* gene leading to insensitivity to AAL-toxins could result in a gene product that is still functional but has less affinity to AAL-toxins. In general, transposon insertions in genes cause loss-of-function mutations. If AAL-toxins cause cell death by direct intervention with the *asc* gene product, then transposon insertions in this gene are expected to be lethal. However, the high frequency by which resistant plants were obtained indicates that EMS caused non-lethal loss-of-function mutations in *asc*.

For insertional inactivation of the *asc* allele, the ET90 tomato transformant was used. The T-DNA of this susceptible (*asc/asc*) line contains a nonautonomous *Ds* transposon and was located 10 cM from the *Asc* locus (Fig. 3). For transactivation of *Ds*, a stabilised *Ac* line was used in the same genetic back-

ground. Following selfing and outcrossing of the F_1 to a susceptible tester stock, progenies are screened for loss of sensitivity to AAL-toxins by seedling assays (Fig. 6). Molecular analysis showed that *Ds* is somatically active and that transposed *Ds* elements are germinally transmitted with a frequency of 7%. Presently, 110,000 progenies have been germinated on AAL-toxins, however, no insensitive plants have been found. The recovery of such insertion mutant depends on the conditions that transposon inactivation of the susceptibility allele, using the *Ds* element at the ET90 position, results in a viable mutant that is insensitive to AAL-toxins. Estimations that include the frequency and distance of transposition predict a mutation rate in the range of 10^{-5} (Van der Biezen et al., 1994c). Based on the mutation frequency and population size it is calculated that a 67% probability of finding an insertion mutant has been reached.

Positional cloning of the Asc locus

To construct a high resolution map of the *Asc* locus, chromosome 3L specific RFLP markers (Tanksley et al., 1992) were hybridised with DNA of F_2 plants derived from an interspecific cross between *L. pennellii* and *L. esculentum* (Van der Biezen et al., 1994a). Two closely linked markers, TG134 (0.7 cM) and TG442 (1.4 cM), were identified at both sides of the *Asc* locus (Fig. 3). The DNA sequence of the RFLP markers was used to design oligomers which were employed in polymerase chain reactions (PCR) to screen a yeast artificial chromosome (YAC) library. For each RFLP marker (TG134 and TG442) a corresponding YAC was isolated: Y-134 (630 kb) and Y-442 (700 kb), respectively. Presently, the YAC end-probes are being isolated to permit each insert to be placed in the correct orientation relative to *Asc*. It remains to be determined whether Y-134 and/or Y-442 contain *Asc* or that additional YACs need to be isolated for the construction of a contig.

To determine the position of the end-probes relative to *Asc*, two sets of plants harbouring chromosomal recombinations at either side of the *Asc* locus are required. The resistant wild tomato *L. pennellii* (*Asc/Asc*) was crossed to a susceptible chromosome 3 tester line (*asc/asc*) containing the markers *baby lea syndrome* (*bls*) and *solanifolia* (*sf*) (Fig. 3) (Van der Biezen et al., 1994a). To increase the efficiency of recombinant selection, first F_2 plants are selected for recombinations between these phenotypical markers. Following visual selection, plants are scored for *Asc* by leaflet bioassays with AAL-toxins. Only plants that are sensitive to AAL-toxins (*asc/asc*) and that either carry a recombination between *asc* and *bls* or between *asc* and *sf* are selected for subsequent DNA analysis. The former plants (*asc*, *Bls*) are molecularly analysed for recombinations between *asc* and TG442, the latter group (*asc*, *Sf*) for recombinations between *asc* and TG134. Since the genetic distance between *Asc* and both morphological markers is approximately 20 cM, the efficiency of recombinant selection is about 5 times higher than without preselection. For the detection of recombinations between *L. pennellii* and *L. esculentum* chromosomes, Southern hybridisation (RFLPs) and PCR techniques (polymorphisms in product size or restriction patterns) are applied on pooled plant selections (Churchill et al., 1993).

Concluding remarks

The fungus *A. a. lycopersici* secretes host-selective AAL-toxins that are the primary molecular determinants of pathogenicity for the Alternaria stem canker disease in tomato. Most likely, the toxins are recognised by the enzyme ceramide synthase which leads to inhibition of the sphingolipid synthesis and results in cell death. The ability to produce AAL-toxins enables the saprophyte to destroy host cells and to obtain nutrients and, hence, allows the pathogen to proliferate and further infect susceptible tissues. Resistance and susceptibility to fungal infection are conceivably the result of the concomitant insensitivity and sensitivity to the AAL-toxins, respectively. Sensitivity to AAL-toxins in tomato is regulated by the *Asc* locus which, consequently, encodes a host recognition factor. Likewise, the interaction between the fungal pathogen and tomato comes down to a direct or indirect interaction between the product(s) of the *Asc* locus and the AAL-toxins. The molecular basis of the specificity of host recognition is determined by the alleles of the *Asc* locus.

It is expected that the physical isolation and characterisation of *Asc* will elucidate its function in the interaction between *A. a. lycopersici* and tomato. To that end, approaches are applied that are based on different characteristics. Product-based strategies depend on the identification of the biochemical target(s) of AAL-toxins. However, ceramide synthase is a very unstable membrane-bound enzyme and therefore difficult to purify. Moreover, this enzyme might not be encoded by *Asc*. A phenotype-based approach such as transposon tagging relies on the ability to detect

the phenotype (susceptibility or resistance) following inactivation of an *Asc* allele by the transposon. For transposon mutagenesis of *Asc* or *asc*, it is crucial that the functional allele is inactivated resulting in a non-lethal and recognisable insertion mutant. Finally, position-based strategies are straightforward and are potentially not dependent on the function or biochemical characteristics of the *Asc* product(s). Following the identification of a YAC or cosmid vector harbouring *Asc*, complementation of resistance or susceptibility can be achieved by transforming susceptible or resistant lines, respectively.

Harmful effects of *A. a. lycopersici* to tomato crops remained limited as yet. No (intentional) selection for resistant plants carrying *Asc* has been carried out during development of commercial tomato varieties. However, some of the present lines are susceptible (*asc/asc*) to fungal infection, indicating that the aggressiveness and/or the occurrence of the pathogen is restricted. Consequently, the question arises as to whether resistance to *A. a. lycopersici* infection, conferred by the *Asc* locus, is a result of adaptation to coexistence with the pathogen. Is the dominant *Asc* allele derived from the recessive *asc* allele? Since the EMS-induced mutations at the recessive *asc* allele result in dominant alleles this might be the case.

The compatible interaction between tomato and *A. alternata* could, alternatively, also be the result of adaptation at the fungal side. A mutation in a gene involved in a biosynthetic pathway of *A. alternata* could result in accumulation of metabolic compounds. Possibly, increased concentrations of certain intermediates could be fortuitously toxic to some plants. The observation that different quantities of toxins are produced among different culture media and different fungal isolates supports this hypothesis. If the sensitive tissues are destroyed by the toxin-producing fungus, a new nutritional source is established. Accordingly, such mutation would change a normally exclusive saprophyte to a facultative saprophyte or plant pathogen.

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